Single-Injection HPLC Analysis of Acids, Sugars, and Alcohols in Cucumber Fermentations

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A method was developed to analyze 11 compounds, which may occur in fresh or fermented cucumbers, by a single injection onto a polymeric sulfonated styrene–divinylbenzene cation ion-exchange column. Isocratic elution with 1.6 mM heptafluorobutyric acid resulted in separation and elution of the following compounds in 28 min: glucose, fructose, glycerol, ethanol, 1-propanol, malic acid, succinic acid, lactic acid, acetic acid, propionic acid, and butyric acid. A conductivity detector for determination of organic acids and a pulsed amperometric detector for analysis of sugars and alcohols were connected in series to analyze these compounds without interferences. Use of internal or external standardization and either peak heights or peak areas for quantitation were all found to give satisfactory analyses for all 11 compounds.

INTRODUCTION

Sugars, acids, and alcohols either are utilized as substrates or appear as products of fermentation in fermented foods such as pickles. Formation or disappearance of certain of these compounds may be indicators of spoilage, while others may not change substantially during fermentation. Rapid, sensitive, and specific quantitative analysis of these compounds has continued to be a difficult analytical problem due to their lack of distinctive chromophoric groups and the similarity of chromatographic behavior of closely related compounds. Marsili (1977) used gas chromatography to measure glucose, fructose, and lactic acid in cucumber juice fermentations. McFeeters et al. (1984) used a polymeric sulfonated styrene–divinylbenzene cation-exchange column with a lead counterion (Aminex HPX-87P) and a C_{18} reversed-phase column in separate runs to analyze sucrose, glucose, fructose, mannitol, malic acid, lactic acid, acetic acid, and ethanol in vegetable fermentations. Làzaro et al. (1989) used an Aminex HPX-87H cation resin in the H^+ form with UV and RI detection in series to analyze glucose, fructose, malic acid, lactic acid, acetic acid, and citric acids in cucumber fermentations with a single injection. Due to overlap in the elution of fructose and malic acid, it was necessary to construct and solve a set of simultaneous equations to obtain reliable concentrations for these two components. McFeeters et al. (1993) developed a procedure to reduce fructose to sugar alcohols with sodium borohydride to determine malic acid with either a UV or RI detector when fructose was present in samples.

To improve the selectivity and sensitivity for the detection of acids and sugars in cucumber fermentations, an effort was made to measure organic acids with a conductivity detector and sugars with a pulsed amperometric detector (PAD). Separation of organic acids by ion exclusion on a Dionex ICE-AS5 column using 1.6 mM heptafluorobutyric acid as the eluant and tetrabutylammonium hydroxide as the suppressor solution (Dionex, 1986) did not give reliable separation of the compounds of interest. This was similar to the results of Kupina et al. (1991), who demonstrated separation of organic acids and detection by conductivity in wine samples with an ion-exchange column (Dionex OmniPac PAX-500). However, the separation required a four-solvent gradient to achieve a suitable resolution. Chromatograms had a rising baseline due to the increasing conductivity of the eluant solutions during a run.

Elution of a polystyrene–divinylbenzene cation column in the protonated form with 0.01 N sulfuric acid (Làzaro et al., 1989) would resolve the acids of interest in cucumber fermentations, provided the detector did not detect sugars or alcohols which coelute with the acids. This type of column has also been widely used for the analysis of acids in wine with a UV detector (Frayne, 1986; Hunter et al., 1991; Schneider et al., 1987). Detection of acids with a conductivity detector would provide greater sensitivity than a UV detector and eliminate the interference problems of an RI detector in cucumber juice or fermentation brines. However, successful use of the conductivity detector required that the baseline conductivity of the eluant solution be reduced postcolumn by an appropriate conductivity suppressor system. Such a suppressor system was not available for an eluant solution containing sulfuric acid. Since a conductivity suppression module was commercially available for heptafluorobutyric acid, sulfuric acid was replaced by 1.6 mM heptafluorobutyric acid as the eluant solution for the cation-exchange column.

The eluant solution after suppression of the baseline conductivity and detection of the organic acids in the conductivity cell was a very dilute acid solution. It was realized that by mixing the eluant stream from the conductivity cell with NaOH solution, the pH could be raised so that sugars eluted from the cation-exchange column could be detected with a PAD placed in-line after NaOH addition. Dionex Corp. has used postcolumn addition of NaOH prior to the PAD for detection of monosaccharides eluted from a Carbopac PA1 column with NaOH solution too dilute to obtain an optimum response with the PAD (Dionex, 1987). Since glucose, fructose, and alcohols present in cucumber fermentations also separate from each other on the cation-exchange resin used for organic acid analysis, these compounds could be analyzed by pulsed amperometric detection during the same chromatographic run as organic acid analysis.

The objective of this paper is to describe a method for single-injection quantitation of acids, sugars, and alcohols in cucumber juice and cucumber fermentation brine. This

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was accomplished by separation on a cation-exchange column in the protonated form using dilute heptafluorobutyric acid as the isocratic eluant solution. A conductivity detector and PAD were connected in series for detection of 11 compounds.

MATERIALS AND METHODS

Chromatography System. The chromatographic system included a Dionex (Sunnyvale, CA) GPM-II gradient pump (No. 42127), a Rheodyne (Cotati, CA) Model 9125 PEEK injector with a 10-μL loop, a Bio-Rad cation guard column (No. 125-0129, Richmond, CA), a Phenomenex ROA organic acid column (No. OOH-0138-KO) placed in a Waters column heater and maintained at 65 °C, a Dionex AMMS-II micromembrane suppressor (No. 38019), a Dionex reagent delivery module RDM (No. 39852) pressurized with helium for delivery of suppressor solution, a Dionex conductivity detector CDM-II (No. 40157), a Dionex 122-cm packed-bead reaction coil (No. 3606), a Dionex pressurizable reservoir chamber (No. 37053), a Dionex PAD with a gold electrode (No. 39553), and a Dionex eluant degas module EDM-II (No. 39852) to maintain the eluant solution under helium pressure. Data collection was done with slaved channels on a two-channel Chrom-1AT board and Lab Calc chromatography software (Galactic Industries Corp., Salem, NH) installed on a Gateway2000 (Sioux Falls, SD) 486/25 computer.

Operating Conditions. Mobile phase for the Phenomenex ROA column was 1.6 mM heptafluorobutyric acid (No. 16 419-4; Aldrich Chemical Co., Milwaukee, WI) in water. Column temperature was maintained at 65 °C. The flow rate was 0.7 mL/min. The conductivity of the eluant was reduced by passing it through the AMMS-II micromembrane suppressor with a 2 mL/min countercurrent flow of 5 mM tetrabutylammonium hydroxide (No. 17 878-0; Aldrich) as the suppressor solution. The conductivity detector was set at 30 μS full-scale with a 0–1-V output. Eluant from the conductivity cell was mixed with 0.3 N NaOH at a flow rate of 0.7 mL/min. NaOH solution was prepared from 50% carbonate-free NaOH (Fisher Chemical Co., Pittsburgh, PA). Mixing of the conductivity cell eluant with NaOH solution was done in the packed-bead reaction coil. The PAD was set with E_1 = +0.05 V, E_2 = +0.6 V, E_3 = −0.8 V, T_1 = 300 ms, T_2 = 120 ms, T_3 = 60 ms, and 3000 nA full-scale (Dionex, 1987).

Sample Preparation. Standard solutions for cucumber juice and cucumber fermentation brine analysis were prepared in water to contain 1 mM malic acid, 1 mM succinic acid, 6 mM lactic acid, 5 mM acetic acid, 5 mM propionic acid, 5 mM butyric acid, 1.2 mM glucose, 1.2 mM fructose, 1 mM glycerol, 50 mM ethanol, and 50 mM 1-propanol. Standard curves were determined by running four dilutions of this mixture with a maximum dilution of 25-fold. *meso*-Erythritol (1.5 mM) and isobutyric acid (8 mM) were added as internal standards to all standard solutions and samples. *meso*-Erythritol served as the internal standard for sugars and alcohols determined by the PAD. Isobutyric acid was the internal standard for the organic acids determined with the conductivity detector.

Cucumber juice was prepared from commercial pickling cucumbers (Daeschel et al., 1988) and frozen until use. Cucumber fermentation brine was prepared by fermentation of cucumbers with *Lactobacillus plantarum*. Cucumbers (38–44-mm diameter) were covered with an equal volume of brine which contained 106 mM acetic acid and 36 mM calcium hydroxide to give an initial pH of 4.6. No salt was added during fermentation. The jars were inoculated with 10^6 CFU/mL *L. plantarum* MOP3 from the laboratory culture collection. After fermentation, 8% NaCl was added to give a fermentation brine with the NaCl concentration similar to that used for commercial salt-stock pickles.

Each compound was added to the cucumber juice and fermentation brine matrices at 11 concentrations from zero to the maximum level cited above in increments of 10% of the maximum. As injected, the cucumber juice was diluted 24-fold and the fermentation brine 12-fold. These dilutions were considered probable minimum dilutions to have the concentrations of the compounds of interest within the analytical range.

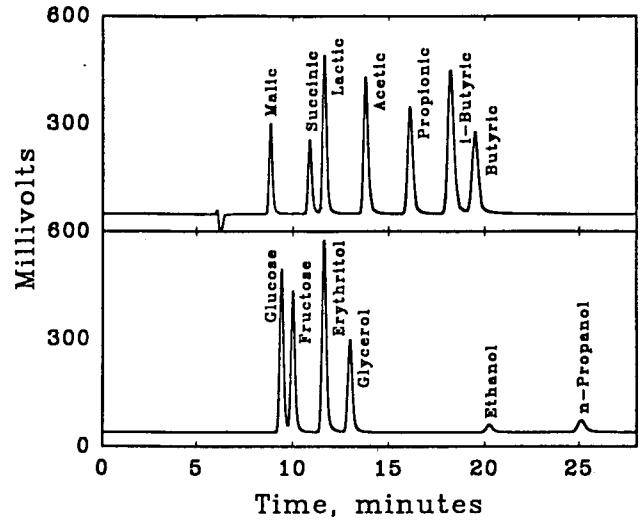

Figure 1. Chromatograms from a single injection of a standard solution of the 11 compounds measured along with the internal standard compounds. The conductivity detector chromatogram is in the upper panel. The chromatogram from the PAD is in the lower panel.

Prior to injection, the samples were centrifuged for 5 min in an Eppendorf microcentrifuge at 15000*g* to remove any particulate matter.

Experimental Design. The experimental design used for this study is based on the existence of a full set of mutually orthogonal 11 × 11 Latin squares. A conceptual array of 121 mixtures consisting of not more than 12 compounds each at 11 different levels, such that every compound appears at some level in each mixture and appears exactly once with every level of each of the other compounds, is possible (in this case only 11 compounds were used). Procedure OPTEX in SAS QC was used to select subsets of 33 mixtures from such an array of 121 possible mixtures. The selection criterion was to find the subset which should maximize the amount of information available on linear relationships between the chromatograms generated and the amounts of the various compounds present in the mixtures. One such subset of 33 mixtures was prepared in cucumber juice, and a different set of 33 mixtures was added to fermentation brine.

To determine if there was a preferred integration method for analysis in these sample matrices, the 66 chromatograms generated were integrated by four methods: (1) peak area/internal standard, (2) peak area/external standard, (3) peak height/internal standard, and (4) peak height/external standard. Data from the 66 chromatograms were read into a SAS data set, and regressions between the added and analyzed quantities of each compound in each sample matrix were calculated with the REGRESSION procedure in SAS STAT.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms from a single injection of a standard solution used for cucumber analysis. Good resolution of the compounds was achieved. Figures 2 and 3 show chromatograms with these compounds added to cucumber juice or fermentation brine, respectively. There were no components present in cucumber juice which interfered with analysis of this group of compounds. Salt in the fermentation brine resulted in a large peak on the conductivity chromatogram (Figure 3), but it did not interfere with the analysis. The only sample preparation required was addition of internal standard solution along with the appropriate amount of water, followed by a brief centrifugation in a microcentrifuge to remove any particles prior to injection. Of the compounds detected with the PAD, glucose and fructose are the soluble sugars present in cucumbers. Glycerol may be formed if yeasts participate in the fermentation. Small amounts of ethanol are formed by cucumber tissue enzymes under anaerobic conditions,

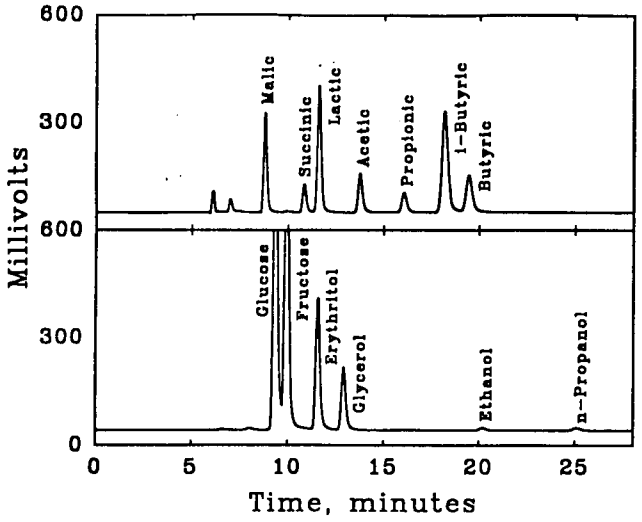

Figure 2. Chromatograms from a single injection of compounds added to or naturally present in cucumber juice.

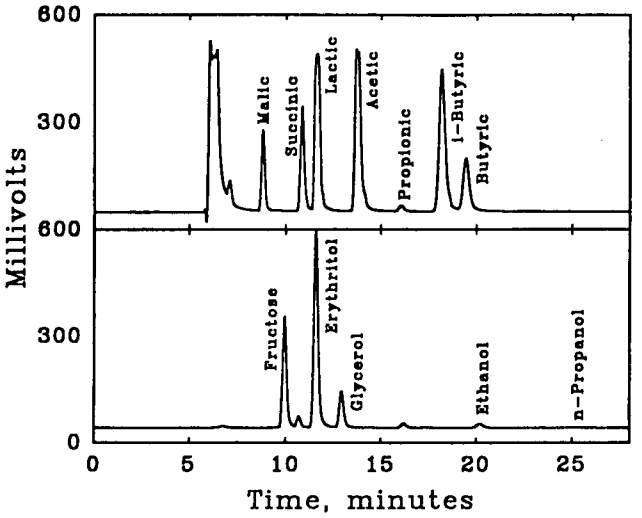

Figure 3. Chromatograms from a single injection of compounds added to or naturally present in cucumber fermentation brine with 4% NaCl.

while larger amounts of ethanol may occur if heterolactic acid bacteria or yeasts participate in the fermentation. Finally, 1-propanol can be formed during anaerobic spoilage of fermented cucumbers (Fleming et al., 1989). The acids included in the analysis were selected because malic acid is the major organic acid to occur naturally in cucumbers. Succinic acid may be formed from malic acid by lactic acid bacteria. Lactic acid is formed from sugar fermentation and from malolactic degradation of malic acid. Acetic acid is commonly added to cucumber fermentation brines to suppress the growth of undesirable microorganisms. Propionic acid and butyric acid can be produced from lactic acid during anaerobic spoilage of salt stock cucumbers (Fleming et al., 1989). The peak height and peak area response factors of these compounds, expressed as mV and mV s for a 10-μL sample with a 1 mM concentration, are presented in Table I.

Figure 4 shows a graph of analyzed vs added malic acid in cucumber juice and in fermented brine when the chromatograms were integrated by peak area with an isobutyric acid internal standard. The slope of the line should be 1.0 for an ideal analysis. The intercept for cucumber juice is 0.57 mM due to the malic acid naturally present in the juice. The slope, intercept, and R^2 for each of the 11 compounds in the two sample matrices for the peak area/internal standard integration method are given

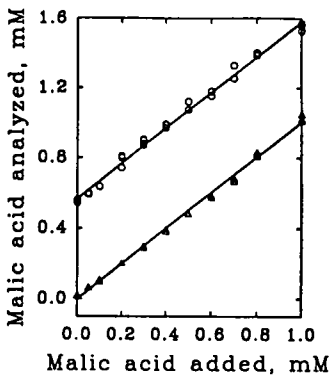

Figure 4. Relationship between added and analyzed malic acid in cucumber juice (○) and fermentation brine (△).

Table I. Response Factors for Compounds Analyzed on Conductivity and Pulsed Amperometric Detectors

compound	peak area/ mM (mV s)	peak height/ mM (mV)
	Conductivity Detector	
malic acid	60.5	296.1
succinic acid	60.3	250.4
lactic acid	22.0	90.1
acetic acid	23.6	79.9
propionic acid	21.9	66.7
butyric acid	21.2	50.4
	Pulsed Amperometric Detector	
glucose	106.1	439.9
fructose	93.6	370.8
glycerol	82.4	290.8
ethanol	0.203	0.494
1-propanol	0.381	0.736

Table II. Compilation of Slopes, Intercepts, and R^2 Values for Peak Area/Internal Standard Integration in Cucumber Juice and Brine[a]

compound	medium	slope mM added/ mM analyzed	intercept mM	R^2	range of slopes[b]
glucose	brine	1.035	−0.001	0.9965	0.030
fructose	brine	0.981	0.014	0.9985	0.020
glycerol	brine	1.006	0.045	0.9997	0.007
ethanol	brine	1.036	2.2	0.9961	0.047
1-propanol	brine	1.049	0.19	0.9954	0.059*
malic acid	brine	1.008	−0.003	0.9975	0.064*
succinic acid	brine	1.099	0.17	0.9966	0.025*
propionic acid	brine	1.026	0.007	0.9964	0.034
butyric acid	brine	1.056	−0.024	0.9957	0.083*
glycerol	juice	1.007	0.013	0.9995	0.022
ethanol	juice	1.066	0.37	0.9947	0.019
1-propanol	juice	1.079	0.6	0.9926	0.018
malic acid	juice	1.014	0.57	0.9939	0.052
succinic acid	juice	1.045	0.005	0.9935	0.010
lactic acid	juice	1.006	0.007	0.9982	0.028
acetic acid	juice	1.016	0.05	0.9991	0.012
propionic acid	juice	1.020	−0.137	0.9980	0.017
butyric acid	juice	1.020	−0.01	0.9947	0.008

[a] Those instances in which a statistically significant difference in slopes occurred ($P \leq 0.05$) are indicated by an asterisk (*). [b] Four integration methods.

in Table II. Also in Table II, the range of the slopes and intercepts over the four integration methods is presented. The slopes obtained from each integration method were compared to the common slope by analysis of covariance. This analysis was done separately for the cucumber juice and fermentation brine samples when compounds could be analyzed in both matrices. There were four instances among the 18 sets of data analyzed in which the slope from one method was statistically different ($P \leq 0.05$) from the common slope. However, in each of the four cases in which this occurred, there was not a significant

difference in slopes for the same compound in the other medium. The magnitude of the differences in slopes was small even when there was a significant statistical difference (Table II). The conclusion was that any of the four integration methods would give a satisfactory result with this chromatographic technique. Positive intercepts occurred when the compound was naturally present in the cucumber juice or brine. On the basis of the peak area/internal standard method intercepts, there was 0.17 mM succinic acid, 0.045 mM glycerol, and 2 mM ethanol in the fermentation brine in addition to 0.57 mM malic acid in the cucumber juice.

Due to the pattern of addition of components to the fermentation brine and cucumber juice samples, there should have been no significant correlation between the analyzed concentration of each compound and the added concentrations of the other 10 compounds unless the presence of 1 compound influenced the analysis of another compound. No significant correlations were found, indicating that, as expected, no significant interactions occurred in the detection of the 11 compounds.

This chromatographic method for the first time allows the separation and analysis of all major components which are degraded or formed during normal and aberrant cucumber fermentations. The interferences of malic acid with fructose, butyric acid with ethanol, and propionic acid with a naturally occurring unknown compound, which have been observed in this laboratory with a refractive index (RI) detector, do not occur with this technique. The unknown compound in cucumber juice and fermentation brines which interferes with propionic acid analysis was not observed with either detector. In the other cases, each compound in a chromatographically nonresolved pair was detected by only one detector.

Sensitivity of detection for sugars and organic acids was considerably greater than with the RI detector or the UV detector set at 210 nm for organic acids. It is likely that greater sensitivity of detection for both sugars and organic acids could be achieved than was required by this application. The sensitivity of detection of 1-propanol and ethanol was several hundredfold less than that of glucose on the PAD (Table I). Despite this low detector sensitivity, the presence of these compounds at low levels could be observed due to the high stability of the baseline. However, accurate measurement of ethanol at the low concentrations usually found in cucumber fermentations would require rerunning a sample with minimum dilution, perhaps with the PAD sensitivity increased. The baseline stability of the detector with this chromatographic system is such that a 10-fold increase in detector sensitivity is reasonable. If higher than normal amounts of ethanol were present due to yeast contamination of the fermentation, for example, the ethanol could be reasonably quantitated with the standard dilution.

This system was quite stable chromatographically, mechanically, and electronically such that it functioned over extended periods with little down time. It should provide the opportunity to develop methods for the simultaneous analysis of monosaccharides, disaccharides, sugar alcohols, alcohols, and organic acids in a number of food applications. Doyon et al. (1991) have discussed some of these possibilities for an RI/UV dual detection system using a chromatographic column similar to that used here. The same chromatographic variables, i.e., temperature and eluant acid concentration, are available in this system. This dual detector method has the advantage that, with rare exceptions, a compound will be detected by only one of the two detectors. An RI detector responds to all of these compounds with the result that more interferences will occur in complex matrices. A UV detector at 210–215 nm responds not only to organic acids but also to many other common food components, such as water-soluble pigments, which absorb UV light and which are not always removed by the guard column. Despite the considerable potential of this dual detector combination, there will certainly be cases in which two or more compounds detected by the same detector will not be resolved from each other given the broad range of naturally occurring compounds and limited chromatographic flexibility of this type of ion suppression chromatography. One case important for analysis of heterolactic acid vegetable fermentations, such as sauerkraut or kimchi, is that fructose and mannitol are not resolved chromatographically and both are detected only with the PAD.

The high sensitivity of these detectors for all of the compounds studied, with the exception of primary alcohols, also means that in many instances food samples may be diluted to a greater extent than is possible with RI or UV detectors. This should lead to extension of the useful lifespan for the columns. The column that was used for this work has been used for over 2500 injections over 18 months without loss of resolution.

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Please note one error in the methods section. The micromembrane suppressor required is the AMMS-ICE suppressor, not the AMMS-II model stated in the article.